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CONTRACTING ORGANIZATION: Florida A&M University
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13. ABSTRACT *(Maximum 200 words)*

Breast cancer is the second leading cause of cancer-related deaths for women. Estrogen metabolism as well as mitochondrial alterations are strongly associated with cancer. We propose that the mitochondrial metabolism of estrogens play a major role in the induction of cancer. We have hypothesized that DES is metabolized by the mitochondria to DES quinone. We have further proposed that diallyl sulfide (DAS), a component of garlic, will inhibit the metabolism of DES. To test the hypotheses we isolated mitochondria, microsomes, and nuclei from the breast tissue of female ACI rats. UV analysis revealed that all three organelles catalyzed the oxidation and reduction of DES. DAS also inhibited these reactions by 50%. These results were confirmed by HPLC analysis. These results suggest that metabolism of estrogens may plays a critical role in estrogen induced breast cancer. DAS and similar chemicals may inhibit the formation of breast cancer by inhibiting this metabolism. This study provides a rationale for more advanced studies on DAS and similar chemicals on the prevention of breast cancer.

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7/30/99

To: Commander
U.S. Army Medical Research and Material Command
ATTN:MCMR-RMI-S
504 Scott Street
Fort Detrick, Maryland 21702-5012

From: Dr. Ronald D. Thomas
Florida A&M University
College of Pharmacy and Pharmaceutical Sciences
Division of Environmental Toxicology

RE: Letter of Propriety

Dear Commander:

This letter is to inform you that the data on pages four and five have not been published. I request that these documents containing these data be protected.

Sincerely,

Dr. Ronald Thomas
Florida A&M Universtiy

FOREWORD

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Ronald Thorn

PI - Signature Date

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Introduction:

Breast cancer is the second leading cause of cancer related deaths for women (MMRW, 1994). Despite intensive research, the mechanism of estrogen-induced cancer is not clearly understood. The most current school of thought is that, estrogens are metabolized to reactive intermediates that induce genetic instability, by binding to DNA and nuclear proteins, thus initiating carcinogenesis (Liehr and Roy, 1990). In concert with initiation, the mitogenic action of estrogen could stimulate the promotion and progression of estrogen-induced cancer (Fishman *et al*, 1995).

Traditionally, metabolic studies are performed with microsomes *i.e.*, endoplasmic reticulum (ER). We propose that reactive metabolites of estrogen are generated in close proximity of their proposed targets, mitochondrial (mt) DNA and nuclear (n) DNA; therefore, the estrogen must be metabolized by the mitochondria and/or the nuclei. In previous studies we have demonstrated that mitochondria and nuclei have the metabolic capability of metabolizing estrogen to reactive intermediates. (Roy and Thomas, 1994; Thomas and Roy 1995).

The study of estrogen-related DNA damage has been focused on nuclear (n)DNA, however mtDNA is the primary site of attack by reactive metabolites of procarcinogens (Perin-Roussel, *et al* 1995) (Balaansky *et al,* 1996). Mitochondrial DNA adducts appear to persist longer than nDNA adducts (LeDoux *et al*, 1992Other mitochondrial alterations such as, gene mutatuons, gene expression, energy metabolism, the amount and forms of mtDNA and mitochondrial calcium metabolism have been associated with tumor cells (Baggetto, 1993). Currently, there is a limited data on the mitochondrial metabolism of estrogens.

We hypothesize that DES is metabolized by the mitochondria to DES quinone. We further propose that diallyl sulfide (DAS), a component of garlic, will inhibit the metabolism of DES. To test these hypothesis we will incubate mitochondria with DES, Cumen hydroperoxides (oxidation cofactor) and/or DAS. The oxidation products will analyzed by UV absorption and HPLC analysis. Reduction reaction systems including mitochondria, DES quinone, and NADH (reduction cofactor) will be analyzed by UV absorption and HPLC. Parallel oxidation and reduction reactions will be carried out with nuclei and microsomes. This metabolic inhibition may play a role in the chemoprevention of estrogen-induced cancer. This data will provide a foundation for further investigation of the chemopreventive properties of DAS and structurally similar compounds.

Body: Progress of experiments for the budget year 1998-1999

The following tasks were proposed for the first budget year:

Task 1. To demonstrate the oxidation and reduction of DES catalyzed by various organelles (mitoplasts, nuclei, and microsomes) and the inhibition of this metabolism by DAS.

1. Organelles (mitoplasts, nuclei and microsomes) will be isolated by differiential centrifugation
2. Ten rats will be needed to isolate adequate amount of organelles from breast tissue.
3. The rats will be dosed with β-napthoflavone, to induce cytochrome p-450s that metabolize estrogen and sacrificed via carbon dioxide exposure.
4. *In vitro* oxidation reactions will be conducted with DES, cumen hydroperoxide (oxidation cofactor) and individual organelles.

5. The oxidation products will be analyzed by UV absorption and HPLC analysis.
6. *In vitro* reduction reactions will be conducted with DES quinone, NADH (reduction cofactor) and individual organelles.
7. The reduction products will be analyzed by UV absorption and HPLC analysis.
8. In parallel experiments, various concentrations of DAS will be added to determine its' inhibitory effects on DES metabolism.

Alterations to original proposal:

The animal model in the original proposal was Noble rats. After reviewing the reviewer's comments and the recommended references it became apparent that female ACI rats are a better model for studying breast cancer. Therefore I used female ACI rats in the following study.

Challenges:

Originally we planned to use 3 – 4 week old rats. However, in these rats the breast tissue was not easily detectable. While attending the American Association for Cancer Research Meeting, it was suggested that I use retired breeders. The mammary glands and breast tissue in these rats was easily dissected and used in experiments. We had problems with our HPLC and were not able to complete analysis on the reduction of DES Q.

Methods:

Animal Treatment:

Ten female ACI rats were treated with β-naphthoflavone (50mg/kg i.p.) daily for four days. The rats were sacrificed by exposure to carbon dioxide. The breast tissue was dissected and homogenized in 5 volumes of 0.25 M sucrose solution containing 1.0 mM phenylmethyl sulfonyl flouride (PMFS). The homogenate was used to isolate nuclei, mitoplasts, and microsomes.

Nuclei Preparation:

The homogenate was filtered through cheesecloth and centrifuged for 10 min at 1,000 x g. The pellet was resuspended in 0.25 M Sucrose and underlayed with 4 volumes of 2.3 M sucrose. The nuclei were pelleted by centrifugation at 100,000 x g for 60 minutes. The nuclei were then washed and resuspended in0.25 M sucrose.

The purity of the nuclei was assessed by both morphological and biochemical analyses. Nuclei were stained with H&E. Phase-contrast microscopy confirmed the presence of intact nuclei. The determination of cytochrome *c* oxidase (Warton, D &, Tzagoloff, A, 1967)an enzymatic marker of mitochondria, showed very low activity (2 μmol/mg protein/min). The activity in purified microsomes ranged form 100- 110 μmol/mg protein/min. Microsomal contamination was assessed by measuring the activity of glucose 6-phosphatase, an enzymatic marker of endoplasmic reticulum (Baginski, *et al* 1974). The activity of glucose 6-phosphatase in nuclei was <1% of that found in microsomes (5.0 pmol/mg protein/min in nuclei versus 563 pmol/mg protein/min in microsomes). This is in agreement with the report of Niranjan *et al (1980)*. These results suggested that nuclei preparations were highly pure.

Mitoplasts Preparation:

The supernatant that remained after the 1,000 x g centrifugation, mentioned in the nuclei preparation section, was used to collect mitoplasts. The mitochjondria was pelleted by

centrifugation at 11,000 x g for 30 minutes. To exclude the possibility of contamination with endoplasmic reticulum, the outer membrane of mitochondria was removed by treating with 1.6 % digitonin. After 30 min of gentle stirring, samples were centrifuged at 11,000 x g for 15 minutes and mitoplasts were collected.

The purity of mitoplasts was assessed by both morphological and biochemical analyses. Mitoplasts were stained with H&E. Phase-contrast microscopy did not reveal any cellular contamination. The determination of cytochrome *c* oxidase (Warton, D &, Tzagoloff, A, 1967), an enzymatic marker of mitochondria, showed 100-110 umol/mg protein/min specific activity. Microsomal contamination was assessed by measuring the activity of glucose 6-phosphatase, an enzymatic marker of endoplasmic reticulum (Baginski *et al* 1974). The activity of glucose 6-phosphatase in mitoplasts was <1% of that found in microsomes (3.0 pmol/mg protein/min in mitoplasts versus 563 pmol/mg protein/min in microsomes). This is in agreement with the report of Niranjan *et al (1980)*. These results suggested that mitoplast preparations were highly pure.

Microsomal Preparation:

The supernantant remaining from the 11,000 x g centrifugation was centrifuged at 40,000 x g for 60 minutes to collect microsomes.

The purity of microsomes was assessed by both morphological and biochemical analyses. Microsomes were stained with H&E. Phase-contrast microscopy did not reveal any cellular contamination. Biochemical analysis for cytochrome c oxidase and glucose 6-phosphatase activity revealed the presence of microsomes with little mitochndrial contamination.

The protein concentration of the organelles (nuclei, mitoplasts, & microsomes) were determined using a BioRAD DC protein assay kit. The organelles were stored at –80 ^{0}C until needed.

Oxidation of DES to DES quinone by mitoplasts and microsomes

The oxidation reaction system contained mitoplasts (0.42 mg equivalent mitochondrial protein) or microsomes (0.346 mg equivalent microsomal protein), 120μM Cumen hydroperoxide (ChP), and various concentrations of DES (0-100 μM) in a final volume of 1 ml 10 mM phosphate buffer, pH 7.5. Some of the reactions were carried out in the presence of Diallyl Sulfide (186 μM and 373μM) The conversion of DES to DES quinone was monitored as a gradual increase in UV absorption in the range of 300-500 nm. The oxidation product formation was also analyzed by HPLC. DES metabolites were extracted by ether. The extraction recovery of synthetic DES quinone was 95 $^{+}$ 5%.

Reduction of DES quinone by mitoplasts and microsomes

The reduction reaction mixture contained mitoplasts (0.42 mg equivalent mitochondrial protein) or (0.35 mg equivalent microsomal protein) 0.5 mM NADH and varying concentrations of DES quinone (0-0.044 mM) in a final volume of 1 ml 10 mM phosphate buffer, pH 7.5. Some of the reactions were carried out in the presence of Diallyl Sulfide (186 μM and 373μM). The reduction of quinone was monitored by its gradual disappearance in UV absorption in the range of 300-500 nm. DES metabolites were extracted ether. At this time no HPLC analysis have been done on the reduction reactions.

Oxidation of DES by nuclei:

The oxidation reaction system contained nuclei (0.45 mg equivalent nuclear protein), 120 μM Cumen hydroperoxide, and 100 μM DES; in a final volume of 1 ml, 10 mM phosphate buffer, pH 7.5. Some of the reactions were carried out in the presence of Diallyl Sulfide (186 μM and 373μM). Due to limited quantity of nuclei only HPLC analysis were performed.

RESULTS:

Mitochondrial Oxidation of DES to DES Q

UV spectroscopy analysis revealed that under oxidation conditions (0.42 mg equivalent mitochondrial protein, 120 μM ChP, 100 μM DES at pH 7.5) the mitoplasts oxidized DES to DES Q. Without the oxidation cofactor, ChP, no DES Q was produced. In the presence of 373μM DAS the oxidation of DES was reduced by 50%. (fig. 1). These results were confirmed by HPLC analysis. The HPLC analysis demonstrated a concentration dependent decrease in the oxidation of DES by DAS. At 186 μM DAS the oxidation was inhibited by 27% and at 373μM DAS the oxidation was inhibited by 50% (Table 1).

Microsomal Oxidation of DES to DES Q

UV spectroscopy analysis revealed that under oxidation conditions (0.346 mg equivalent microsomal protein, 120 μM ChP, 100 μM DES at pH 7.5) the microsomes oxidized DES to DES Q. Without the oxidation cofactor, ChP, no DES Q was produced. In the presence of 373μM DAS the oxidation of DES was reduced by 45%. (Data not shown). These results were confirmed by HPLC analysis. The HPLC analysis demonstrated a concentration dependent decrease in the oxidation of DES by DAS. At 186 μM DAS the oxidation was inhibited by 35% and at 373μM DAS the oxidation was inhibited by 52% (Table 2).

Nuclear Oxidation of DES to DES Q

HPLC analysis revealed that under oxidation conditions (0.52 mg equivalent nuclear protein, 120 μM ChP, 100 μM DES at pH 7.5) the nuclei oxidized DES to DES Q. Without the oxidation cofactor, ChP, no DES Q was produced. The HPLC analysis demonstrated a concentration dependent decrease in the oxidation of DES by DAS. At 186 μM DAS the oxidation was inhibited by 40% and at 373μM DAS the oxidation was inhibited by 60% (Table 3).

Mitochondrial reduction of DES Q

UV spectroscopy analysis revealed that under oxidation conditions (0.42 mg equivalent mitochondrial protein, 120 μM ChP, 100 μM DES at pH 7.5) the mitoplasts oxidized DES to DES Q. Without the oxidation cofactor, ChP, no DES Q was produced. In the presence of 373μM DAS the oxidation of DES was reduced by 50%. (fig. 2).

Microsomal Reduction of DES Q

UV spectroscopy analysis revealed that under oxidation conditions (0.346 mg equivalent microsomal protein, 120 μM ChP, 100 μM DES at pH 7.5) the microsomes oxidized DES to DES Q. Without the oxidation cofactor, ChP, no DES Q was produced. In the presence of 373μM DAS the oxidation of DES was reduced by 45%. (Data not shown). These results have not yet been confirmed by HPLC analysis. (Table 2).

Enzyme Kinetics of Mitochondrial and Microsomal Oxidation Reactions

The rate of DES Q formation in the presence of mitoplasts were dependent of the concentration of DES (fig 3), and a Lineweaver-Burk plot of rate of formation of DES Q and various DES concentrations yielded a k_m of 35.7 μM and a V_{max} of 3.45 nM/ min/mg protein. The Lineweaver-Burk plot of rate of formation of DES Q, various concentrations of DES and two concentrations of DAS (186 μM & 373 μM) revealed that DAS inhibited the oxidation of DES in a noncompetitive manner. As the inhibitior (DAS) concentration increased the k_m remained constant whereas, the V_{max} decreased (fig 3). The V_{max} for the DAS concentrations (0 μM, 186 μM & 373 μM) are as follows : 3.45 nM/ min/mg protein, 2.44 nM/ min/mg protein, and 1.82 nM/ min/mg protein.

The rate of DES Q formation in the presence of mitoplasts were dependent of the concentration of DES (fig 4), and a Lineweaver-Burk plot of rate of formation of DES Q and various DES concentrations yielded a k_m of 80 μM and a V_{max} of 5.56 nM/ min/mg protein. The Lineweaver-Burk plot of rate of formation of DES Q, various concentrations of DES and two concentrations of DAS (186 μM & 373 μM) revealed that DAS inhibited the oxidation of DES in a noncompetitive manner. The k_m remained constant at various inhibitor (DAS) concentrations whereas the V_{max} decreased (fig 4). The V_{max} for the DAS concentrations (0 μM, 186 μM & 373 μM) are as follows of 5.56 nM/ min/mg protein, 4.16 nM/ min/mg protein, and 3.33 nM/ min/mg protein.

Enzyme Kinetics of Mitochondrial and Microsomal Reduction Reactions

The results from the reduction of DES Q to DES was similar to the oxidation reactions. The rate of DES Q disappearance in the presence of microsomes and/or mitoplasts was dependent of the concentration of DES Q. It was also demonstrated that DAS inhibited the reduction of DES Q in a noncompetitive manner as determined by a Lineweaver-Burk plot of rate of the disappearance of DES Q at various concentrations of DES Q and two concentrations of DAS (186 μM & 373 μM) (Data not shown). The kinetic constants for all oxidation and reduction reactions are summarized in table 4.

Research Accomplishments:

- We have demonstrated that mitochondria, nuclei, and microsomes isolated from breast tissue can catalyze oxidation and reduction reactions of DES by UV spectroscopy.
- We have demonstrated that DAS inhibits the oxidation and reduction reactions catalyzed by mitochondria, nuclei, and microsomes by UV spectroscopy.
- The UV analysis of the oxidation reactions were confirmed by HPLC.
- Kinetic constants for the oxidation and reduction reactions were determined.
- The kinetics of the inhibited reactions revealed that the nature of the inhibition is noncompetitive.

Reportable Outcomes:

None at this time

Conclusion:

We have demonstrated that Organelles (mitochondria, microsomes, and nuclei) isolated from the breast of female ACI rats catalyze the oxidation and reduction of DES. This redox-cycling has been demonstrated to produce reactive oxygen species such as Superoxide radicals and DES quinone. The reactive molecules can cause DNA damage and ultimately mutations that can cause cancer. The demonstration of redox-cycling of DES by mitoplasts and nuclei are of significance in that these are not traditional organelles of metabolic study. However, they contain the most critical macromolecule (DNA) in regards to cancer formation. In addition to demonstrating that these organelles can metabolize DES to reactive intermediates, we have demonstrated that diallyl sulfide, a natural component of garlic, inhibits this metabolism in a noncompetitive fashion in all three organelles. This inhibition may help explain the mechanism of the chemopreventive actions of diallyl sulfide. The results of this study will help elucidate the mechanism of estrogen induced breast cancer. This metabolic inhibition may play a role in the chemoprevention of estrogen-induced cancer. This data will provide a foundation for the further investigation of the chemopreventive properties of DAS and structually similar compounds.

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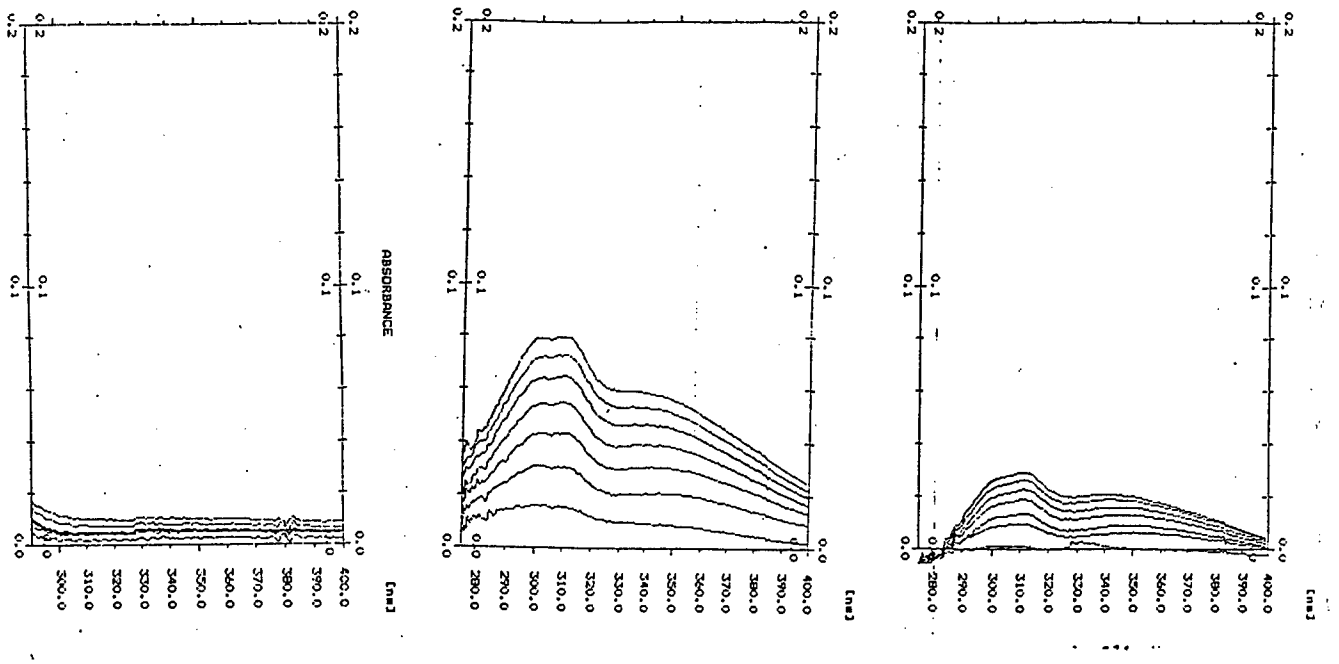

FIG. 1. Oxidation of DES to DES Q catalyzed by mitoplasts. Panel A represents the control reaction *i.e.* contains no cumen hydroperoxide. Panel B represents the complete reaction. Panel C represents the complete reaction with 373 μM DAS. The oxidation was monitored by U.V. Spectroscopy. The lowest absorbances were recorded at time 0. An increase in absorbance was recorded every 30 seconds.

Table 1: MITOCHONDRIAL OXIDATION OF DES AND ITS INHIBITION BY DAS

CONDITIONS	OXIDATION PRODUCTS (DES Q) pM/min/mg protein	%INHIBITION
Control (-ChP)	>5	-0-
Complete System	245 ± 16	-0-
+ DAS (186μM)	166 ± 14	27%
+DAS (373μM)	123 ± 12	50%

The reaction system consisted of 420μg/ml mitoplast protein, 120μM Cumen Hydroperoxide (ChP), 100μM DES in a final volume of 1 ml 10mM potassium phosphate buffer, pH 7.5. Some of the reactions were carried out in the presence of Diallyl Sulfide (DAS). The rearrangement product of DES quinone, Z,Z-dienstrol, was analyzed by HPLC. Each value is a mean of three or four experiments + or – SD.

Table 2: MICROSOMAL OXIDATION OF DES AND ITS INHIBITION BY DAS

CONDITIONS	OXIDATION PRODUCTS (DES Q) pM/min/mg protein	%INHIBITION
Control (-ChP)	>5	-0-
Complete System	312 ± 20	-0-
+ DAS (186μM)	204 ± 15	35%
+DAS (373μM)	149 ± .12	52%

The reaction consisted of 346μg/ml microsomal protein, 120μM Cumen Hydroperoxide (ChP), 100μM DES in a final solution of 1 ml 10mM potassium phosphate buffer, pH 7.5 (Complete System). Some of the reactions were carried out in the presence of Diallyl Sulfide (DAS). The rearrangement product of DES quinone, Z,Z-dienstrol, was analyzed by HPLC. Each value is a mean of three or four experiments + or – SD.

Table 3: NUCLEAR OXIDATION OF DES AND ITS INHIBITION BY DAS

CONDITIONS	OXIDATION PRODUCTS (DES Q) pM/min/mg protein	%INHIBITION
Control (-ChP)	>5	-0-
Complete System	196 ± 38	-0-
+ DAS (186μM)	117 ±14	40%
+DAS (373μM)	78 ± 20	60%

The reaction system consisted of 450 μg/ml nuclear protein, 120μM Cumen Hydroperoxide (ChP), 100μM DES in a total volume of 1 ml 10mM potassium phosphate buffer, pH 7.5. Some of the reactions were carried out in the presence of Diallyl Sulfide (DAS). The rearrangement product of DES quinone, Z,Z-dienstrol, was analyzed by HPLC. Each value is a mean of three or four experiments + or – SD.

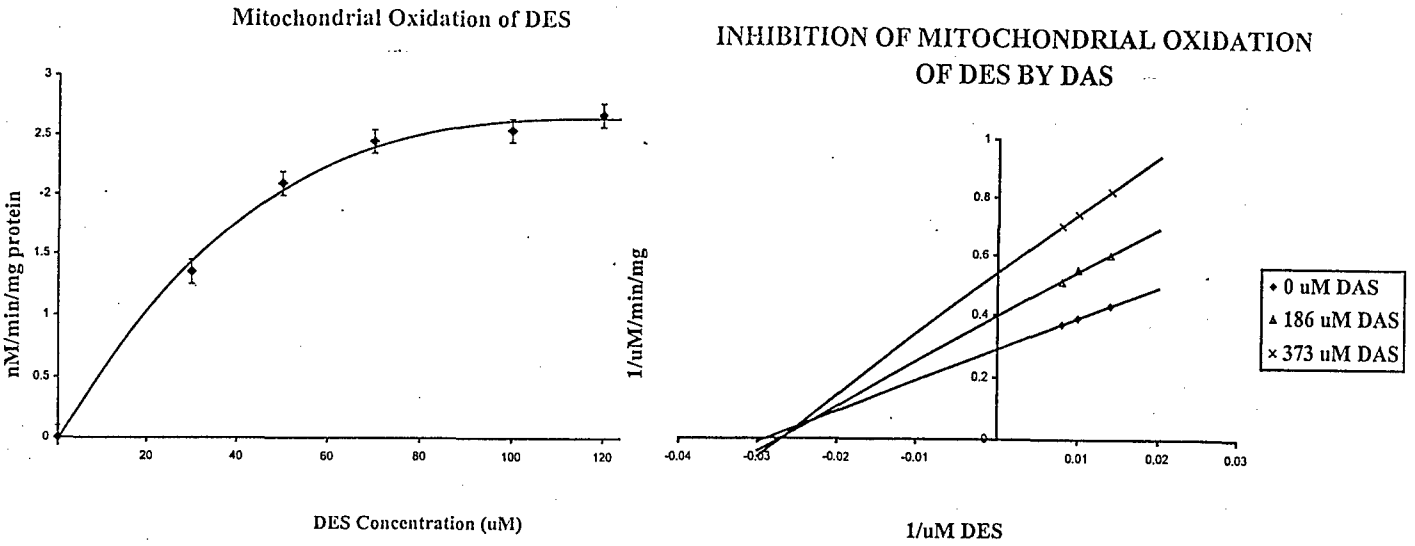

FIG. 2. Influence of various substrate concentrations (A) on the rate of oxidation of DES to DES quinone by mitoplasts. The reaction mixture consisted of purified mitoplasts from β-napthflavone-treated female ACI rats (0.42 mg equivalent protein), 120 μM cumene hydroperoxide, and various concentrations of DES (0-100 μM) in a final volume of 1 ml 10 mM phosphate buffer, pH 7.5. The oxidation was monitored by both UV spectroscopy The data are presented as a double reciprocal plot as substrate-dependent product formation and product inhibition by various concentrations of DAS (B). Values represent the means of three or four experiments.

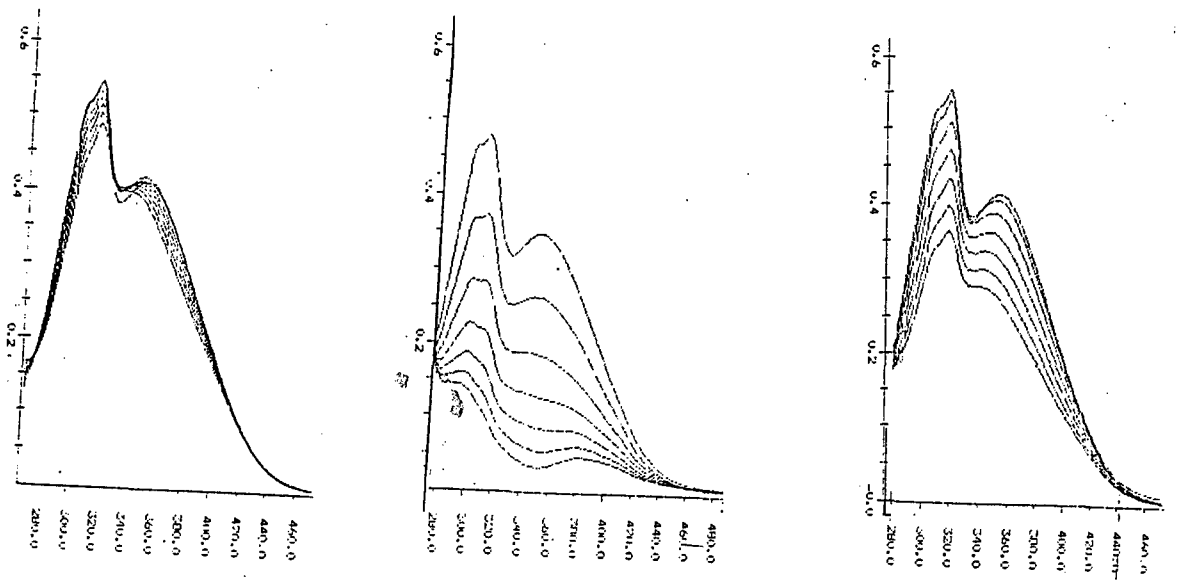

FIG. 3. Reduction of DES Q to DES was catalyzed by mitoplasts. Panel A represents the control reaction i.e. contains no NADH. Panel B represents the complete reaction. Panel C represents the complete reaction with 373 μM DAS. The reduction was monitored by UV spectroscopy. The highest absorbances were recorded at time 0. A decrease in absorbance was recorded every 30 seconds.

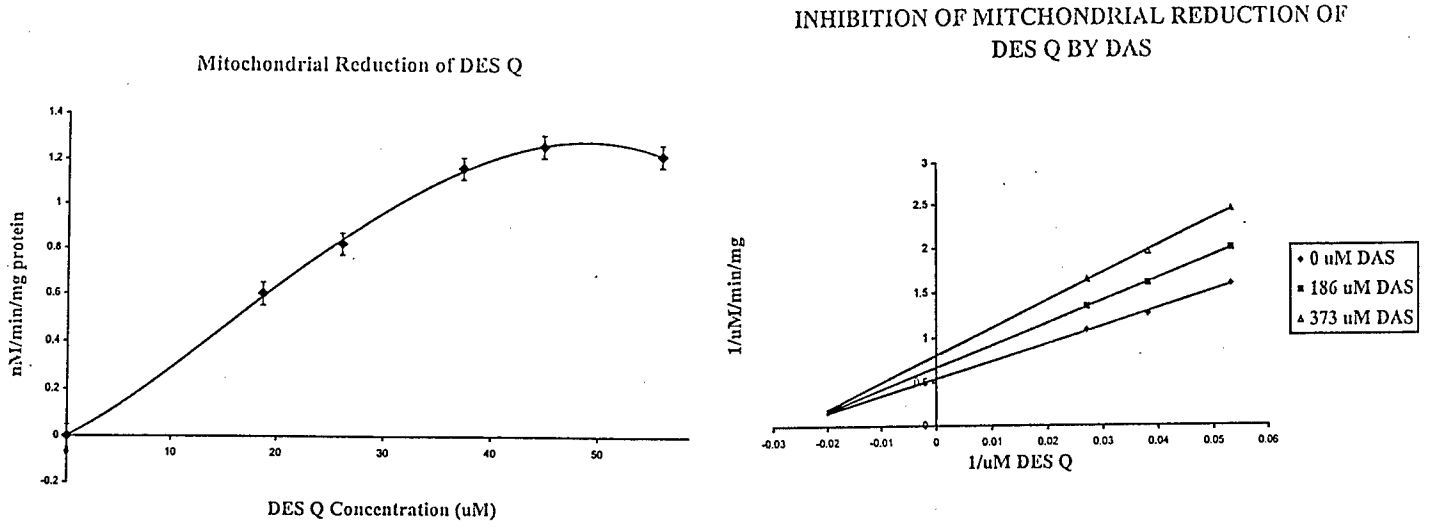

FIG. 4. Influence of various concentrations on the reduction of DES quinone catalyzed by mitoplasts in the presence NADH. The reduction reaction mixture contained purified mitoplasts from female ACI rats (0.42 mg equivalent protein), 50 μM NADH, and various concentrations of DES quinone (0-44 μM) in a final volume of 1 ml 10 mM of phosphate buffer, pH 7.5. The reduction was monitored by both UV spectroscopy The data are presented as a double reciprocal plot as substrate-dependent product formation and product inhibition by various concentrations of DAS (B). Values represent the means of three or four experiments.

Table 4; KINETIC CONSTANTS OF OXIDATION AND REDUCTION REACTIONS

	Oxidation		Reduction	
	Microsomes	Mitochondria	Microsomes	Mitochondria
Km	80 uM	35.7 uM	100 uM	50 uM
$Vmax_0$ 0 uM das	5.56 p.mol	3.45 p.mol	12 p.mol	2.0 p.mol
$Vmax_I$ 186 uM das	4.16 p.mol	2.44 p.mol	14 p.mol	1.5 p.mol
$Vmax_{II}$ 373 uM das	3.33 p.mol	1.82 p.mol	22 p.mol	1.25 p.mol

This table summarizes the oxidation and reduction kinetic constants for microsomes and mitoplasts.

DEPARTMENT OF THE ARMY
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 26 Nov 02

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART
Deputy Chief of Staff for
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